Impaired Expression of Ciliary Neurotrophic Factor in Charcot-Marie-Tooth Type 1A Neuropathy

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INTRODUCTION

Charcot-Marie-Tooth type 1A (CMT1A) disease is the most common form of hereditary neuropathy. It is caused by a duplication of chromosome 17p11.2, which contains the peripheral myelin protein 22 (PMP22) gene. Studies of Pmp22 transgenic (Pmp22tg) animal models indicate that overexpression of this protein is responsible for CMT1A (1). The hallmarks of human and experimental CMT1A are severe, slowing of nerve conduction velocity, demyelination, and hypertrophic changes in peripheral nerves (1, 2). Both in humans and in Pmp22tg animals, however, clinical worsening over time is mainly due to length-dependent axonal atrophy (3, 4). Long-term dorsal root ganglia (DRG) cultures from CMT1A rats also undergo axonal impairment over time (5). Although it is known that axonal loss and neurogenic atrophy of muscle are secondary to demyelination, the mechanisms underlying these phenomena in this disease are not understood (3, 6).

Recently, an antiprogestrone agent (onapristone) and ascorbic acid proved to be effective for the treatment of experimental CMT1A by reducing Pmp22 overexpression (7, 8). The observation that onapristone improves axonal loss and muscle atrophy in the absence of a significant increase in myelin thickness suggests that axonal damage in CMT1A may be exclusively due to dysfunction of Schwann cells (SCs) (9). Indeed, several studies have demonstrated that trophic support from SCs is fundamental to axonal development and survival (10). We recently showed by cDNA microarray analysis that the gene coding for the SC-derived ciliary neurotrophic factor (CNTF) is the most downregulated one in CMT1A rats (11). Moreover, Cntf mRNA is also markedly downregulated in sural nerves of CMT1A patients and in the sciatic nerve of Pmp22tg mice (11, 12). Studies in the Trembler mouse, which carries a point mutation in the Pmp22 gene, also show a significant and selective reduction of Cntf mRNA in peripheral nerves (13). Thus, the loss of CNTF may be involved in the pathogenesis of experimental and human CMT1A.

Ciliary neurotrophic factor is one of the most important neurotrophic factors produced by myelinating SCs, particularly for the preservation of specific axonal structures at the nodes of Ranvier (14, 15). It may also exert a protective effect in demyelinating diseases by preventing apoptosis of oligodendrocytes and enhancing myelin formation (16, 17).

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Online-only color figures are available at http://www.jneuropath.com
In this study, we used a transgenic model of CMT1A to determine whether downregulation of CNTF contributes to the defective myelination properties of Pmp22 overexpressing SCs and whether this downregulation may play a role in the development of the axonal atrophy in CMT1A. We performed sciatic nerve crush experiments to test the capacity of CMT1A SCs to produce CNTF in vivo and to determine the contribution of CNTF to regeneration in CMT1A rats. We also studied the molecular cascade downstream of the CNTF receptor to understand whether downregulation of CNTF in CMT1A rats also affects the classical pathway through which this trophic factor is known to exert its protective effects. Finally, to test the therapeutic potential of CNTF in experimental CMT1A, we treated CMT1A DRG cultures chronically with rat recombinant CNTF (rCNTF) and analyzed the effects on myelination and axonal maintenance.

MATERIALS AND METHODS

Human Samples

Sural nerves biopsies from a 60-year-old man with CMT1A and a 55-year-old man who underwent a biopsy for suspected peripheral neuropathy and whose nerve was normal by morphological and morphometric criteria were used. Immunohistochemical examination on these biopsy samples was performed with informed consent and following all guidelines for experimental investigation of human subjects required by the Department of Neurosciences, Ophthalmology and Genetics, University of Genoa, Italy.

Animal Models

Transgenic rats that overexpress PMP22 (CMT1A rats) were obtained from the colony available in our laboratory (18). The rats were genotyped by polymerase chain reaction (PCR) and both heterozygous (+/−) and homozygous (+/+ ) animals were used for the experiments (18). Normal age-matched littermates were used as controls. Rearing conditions were consistent with the guidelines of the Italian Health Ministry relating to the use and maintenance of transgenic organisms. The experimental procedures were performed in accordance with the international standards on animal welfare and were approved by the Animal Care and Use Committee of the National Cancer Research Institute, Genoa, Italy. Sciatic nerves from Pmp22−/− (“knockout”) and Pmp22+/+ (increased Pmp22 copy number) mice (19, 20) and from Myelin protein zero (Mpz) heterozygous (Po+/−) and homozygous (Po−/−) null mice (21) were also used.

Cell Cultures

Primary SC Cultures

Schwann cells were isolated from sciatic nerves of 30-day-old homozygous (+/+) and heterozygous (+/−) killed CMT1A rats according to a technique optimized for adult animals (22). Wild-type rats from the corresponding genetic background were used to obtain control cultures. Sciatic nerves were dissected under sterile conditions from the rats, minced into small pieces, centrifuged, and transferred to RPMI 1640 medium (Invitrogen, Italy) containing 15% fetal calf serum (Invitrogen), 1.25 U/mL Dispase (crude; Boehringer Mannheim, Germany), 0.05% collag enase (Sigma-Aldrich, Italy), and 0.1% hyaluronidase (Sigma-Aldrich) for 3 hours at 37°C. Digestion products were further mechanically dissociated, rinsed with serum containing medium, and plated on collagen-coated dishes. Schwann cells were grown for 5 days in Dulbecco’s modified Eagle medium/F12 (Invitrogen) containing 10% fetal calf serum (Invitrogen) in the presence of cytosine arabinoside (Sigma-Aldrich) at a final concentration of 10−5 M to produce cultures that were 95% pure. After treatment with cytosine arabinoside, the medium was replaced with basal medium, and 24 hours later, the cells were processed for CNTF quantification as previously described (11).

DRG Cultures

Myelinating DRG cultures were established from 15-day-old embryos, as previously described (23). Embryos were genotyped from the remaining tissue, and the code was broken after quantitative evaluation of each culture. After extracting them using a sterile technique, 30 to 35 DRG were removed from each embryo, pretreated with trypsin (Hanks solution 0.25%), and triturated to provide a suspension of DRG cells in a medium supplemented with 15% bovine calf serum and nerve growth factor 5-μg/mL final dilution. This suspension was plated on a collagen substrate in flexible molded plastic Aclar dishes, and 15 × 10⁴ cells were placed in each dish. Dorsal root ganglia cultures were grown for 30 days in the presence of rCNTF 0.5 μg/mL. R recombinant CNTF was prepared in 0.1% bovine serum albumin in phosphate-buffered saline (PBS), and this buffer was added to the DRG cultures that we used as control in the CNTF-treatment experiments. For each embryo, we established 4 cultures; 2 were treated with rCNTF and the others were treated with the vehicle to have sufficient material for the molecular biology and light microscopy evaluations. The optimal dosage of rCNTF was based on the literature (17). Moreover, preliminary experiments were performed on wild-type cultures treating them for 30 days with 0.1, 0.5, 1, and 10 ng/mL of rCNTF and evaluating the myelin appearance (data not shown). Finally, a dose-response experiment for rCNTF on CMT1A cultures was performed, monitoring the expression levels of nonphosphorylated (SMI32) neurofilaments (NFs) as a marker of axonal injury.

Antibodies

Commercially available monoclonal antibodies against nonphosphorylated (SMI32) NFs (Sternberger Monoclonals, Baltimore, MD), total NFs (52, Sigma-Aldrich), CNTF (R&D Systems, Minneapolis, MN), amyloid precursor protein A4 (AAP) (22C11, Chemicon International, Temecula, CA), β-tubulin (clone TUB2.1; Sigma-Aldrich), glial fibrillar acidic protein (GFAP) (G3893, Sigma-Aldrich), and polyclonal antibodies against phosphorylated signal transducer and activator of transcription 3 (pSTAT3) [tyr-705] and STAT3 (Cell Signalling Technology, Beverly, MA) were used in these experiments. Secondary antibody reactions were carried out using a horseradish peroxidase-conjugated
Semithin sections were stained with toluidine blue. Morphometric studies, samples were fixed in 2.5% glutaraldehyde, dissected from the animal, frozen in liquid nitrogen-cooled scriptase PCR analysis, the nerve stumps were quickly recovered from each rat were used as a control. For real-time reverse transcriptase PCR, the nerve stumps were quickly loosened from connective tissue and crushed with N5 Du- mont forceps 7 mm, previously frozen in liquid nitrogen, incision was made in the right thigh and the gluteus muscle was detached from the sciatic nerve. The nerve was carefully loosened from connective tissue and crushed with N5 Dumont forceps 7 mm, previously frozen in liquid nitrogen, from the sciatic notch. The nerve was crushed twice for 15 seconds; this caused all axons to degenerate but allowed axonal regeneration. At 1 week and 3 months after nerve injury, the distal and proximal nerve stumps were recovered for experimental analyses. The uninjured sciatic nerves of each rat were used as a control. For real-time reverse transcriptase PCR analysis, the nerve stumps were quickly dissected from the animal, frozen in liquid nitrogen-cooled isopentane, and stored at −80°C. For morphological and morphometric studies, samples were fixed in 2.5% glutaraldehyde in cacodylate buffer and embedded in Epon. Semithin sections were stained with toluidine blue.

Sciatic Nerve Crush

Two-month-old wild-type and heterozygous (+/−) CMT1A rats were anesthetized with halothane (Halocarbon Labs, Augusta, SC), followed by 1 mL/kg of a mixture of 80 mg/mL ketamine (Sigma-Aldrich) and 16 mg/mL xylazine (Rompum-Mobay, Shawnee, KS) in sterile saline. A small incision was made in the right thigh and the gluteus muscle was detached from the sciatic nerve. The nerve was carefully loosened from connective tissue and crushed with N5 Dumont forceps 7 mm, previously frozen in liquid nitrogen, from the sciatic notch. The nerve was crushed twice for 15 seconds; this caused all axons to degenerate but allowed axonal regeneration. At 1 week and 3 months after nerve injury, the distal and proximal nerve stumps were recovered for experimental analyses. The uninjured sciatic nerves of each rat were used as a control. For real-time reverse transcriptase PCR analysis, the nerve stumps were quickly dissected from the animal, frozen in liquid nitrogen-cooled isopentane, and stored at −80°C. For morphological and morphometric studies, samples were fixed in 2.5% glutaraldehyde in cacodylate buffer and embedded in Epon. Semithin sections were stained with toluidine blue.

Western Blot Analysis: SM132, APP, GFAP

Total lysates of sciatic nerves and DRG cultures from wild-type and CMT1A rats were prepared directly in 70 μL of BUST buffer (0.5% sodium dodecyl sulfate, 8 M urea, 2% β-mercaptoethanol, 0.01% protease inhibitor cocktail, 0.1 M Tris-HCl, pH 6.8). Total proteins content was determined by the Bio-Rad Protein Detection kit (Bio-Rad), and equal protein amount from normal and transgenic animals was loaded on a 7.5% polyacrylamide gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to 0.2-μm-pore-sized nitrocellulose membranes (Amersham, Arlington Heights, IL) and detected with primary antibodies to APP, different NF isoforms, or GFAP overnight and with anti-β-tubulin antibody for 1 hour. We visualized proteins using the enhanced chemiluminescence detection method (Amersham). Band intensity was measured on the film by a Gel Doc 1000 imaging system (Bio-Rad). The amount of each NF isoform was expressed relative to β-tubulin, which was used as an internal control.

STAT3 Activation

Sciatic nerve and SC protein extracts were prepared in 1× Lysis Buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethyleneglycolctetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/mL Leupeptin) plus 1 mM phenylmethanesulfonyl fluoride, and protein levels were quantified using bicinchoninic acid protein assay (Bio-Rad). Samples were run on polyacrylamide gels and transferred to nitrocellulose membranes. For STAT3/pSTAT3−paired immunoblots, protein samples were loaded in duplicate on separate gels and immunoblotted under identical conditions. Primary antibodies (Cell Signalling Technology) were applied at 1:1000 dilution at 4°C overnight. Signals were visualized using the enhanced chemiluminescence detection method (Amersham), and band intensity was measured on film by a Gel Doc 1000 imaging system (Bio-Rad).

Sciatic nerves from wild-type and CMT1A rats were fixed in 4% paraformaldehyde in sodium cacodylate 0.025 M for 18 hours at room temperature. Fixed nerves were embedded in paraffin. Antigen retrieval was performed by bringing the 5-μm nerve sections to a boil in 10 mM sodium citrate buffer, pH 6.0, and then maintaining them at a sub-boiling temperature for 10 minutes. The sections were then incubated in 3% hydrogen peroxide for 10 minutes and blocked with 5% normal goat serum (NGS) in PBS with Tween-20 (1× PBS/0.1% Tween-20) for 1 hour at room temperature. STAT3 antibody (Cell Signalling Technology) was diluted in 5% NGS–PBS with Tween-20 and incubated overnight at 4°C. Sections were washed in PBS, and biotinylated secondary antibody diluted in 1× Tris-buffered saline/0.1% Tween-20 was applied, followed by avidin-biotin horseradish peroxidase complex (Bio-Genex, Menarini Diagnostics, Firenze, Italy). Reactions were detected with 2,3-diaminobenzidine (Bio-Genex).

Primary SC cultures from wild-type and CMT1A rats, grown on poly-L-lysine-coated coverslips, were rinsed with PBS and then fixed for 15 minutes with 4% formaldehyde in PBS. Cells were permeabilized with ice-cold 100% methanol for 10 minutes at −20°C. Slides were blocked for 1 hour at room temperature and then incubated with pSTAT3 antibody (Cell Signalling Technology) overnight at 4°C. Secondary antibody reaction was performed using Alexa Fluor 488 Goat Anti-Rabbit antibody for 1 hour at room temperature. Schwann cell nuclei were stained with 4’,6-diamidino-2-phenylindole and slides sealed by painting around edges of coverslips with nail polish.

Real-Time Reverse Transcriptase PCR

Total RNA was extracted from distal stumps of uninjured and crushed sciatic nerves of CMT1A rats and their normal littermates to analyze the levels of mRNA coding for Cntf and from sciatic nerves and primary SC cultures to analyze mRNA levels coding for the Cntf receptor subunits (the
membrane-spanning 130-kDa glycoprotein [Gp130], the glycosylphosphatidylinositol [GPI]-anchored CNTF receptor α [Cnfrα] and the LIF receptor [Lifr]) and the different interleukin (IL-6) family members [IL-6, IL-11, leukemia inhibitory factor [LIF], oncostatin M [OSM], cardiotoxin-1 [CT-1], cardiotoxin-like cytokine [CLC], also designated novel neurotrophin-1/B-cell-stimulating factor-3 [Nnt-1/BSF-3]]. Gp130, membrane-spanning 130-kDa glycoprotein; GPI, glycosylphosphatidylinositol-anchored CNTF receptor α (Cnfrα); LIFR, LIF receptor; RT-PCR, reverse transcriptase polymerase chain reaction.

### Light Microscopic Morphometry

Distal stumps from unlesioned and crush-lesioned sciatic nerves of CMT1A and wild-type rats were removed and immediately fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, for 24 hours. The samples were embedded in Epon and prepared for semithin sections, which were stained with toluidine blue. Morphometric analysis was performed in wild-type (n = 3) and CMT1A (n = 3) rats on at least 300 myelinated fibers/animal from both unlesioned and crush-lesioned nerves. From each nerve fascicle, 20 frames were randomly selected at a 1,000× magnification to cover a 0.01-mm² area of each nerve; these were digitalized and stored using the Image Pro-Plus Software (Immagini e Computer, Rho, Milan, Italy). Data from fibers with evident degeneration signs or that have been sectioned in a longitudinal way were not collected. For each myelinated fiber, the outer perimeter and axon perimeters were traced, automatically derived from a circle of corresponding area. Frequency distribution histograms were then calculated for fiber diameter, axon diameter, and myelin thickness. The g-ratios (diameter of the axon divided by diameter of the myelinated fiber) were calculated and plotted as a function of the axonal diameter.

### Immunohistochemistry

Human sural nerve biopsies and rat sciatic nerves were fixed in 4% paraformaldehyde in sodium cacodylate 0.025 M for 18 hours at room temperature. Fixed nerves were embedded in paraffin. Five-micrometer-thick sections were digested with trypsin for 15 minutes at 37°C and then incubated with 10% NGS in PBS for 15 minutes. Anti-CNTF and anti-GFAP antibodies were diluted in PBS containing 1% NGS and incubated overnight at 4°C. Sections were washed in PBS, and biotinylated secondary antibodies were applied, followed by avidin-biotin horseradish peroxidase complex (Bio-Genex). Reactions were detected with 2,3-diaminobenzidazone.

### Table 1. Primer Sequences Used for Real-Time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (Forward)</th>
<th>Primer Sequence (Reverse)</th>
<th>Amplicon Size, bp</th>
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<tr>
<td>CNTF</td>
<td>gCAAACACCTCTATCCCTTC</td>
<td>ACggTAAGCTTTGGTT</td>
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<td>CNTFrox</td>
<td>TggCTATGCTTGAAAGAGAT</td>
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<td>LIFR</td>
<td>CCAAACCTGTGACCCTTCAG</td>
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<td>Gp130</td>
<td>gCAAAGAGATGCCTGTTGAG</td>
<td>TgAATCCGTTGACATCTA</td>
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<tr>
<td>IL-6</td>
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<td>ACCATCCTGATACTACGCTG</td>
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<td>LIF</td>
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<td>IL-11</td>
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<td>CT-1</td>
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<td>rRNA18S</td>
<td>ggggCCCGAGAGCTTTACT</td>
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### Notes

- IL-6, interleukin-6 family of structurally related hematopoietic and neuroepoietic cytokines (IL-6, IL-11, ciliary neurotrophic factor [CNTF], leukemia inhibitory factor [LIF], oncostatin M [OSM], cardiotoxin-1 [CT-1], cardiotoxin-like cytokine [CLC], also designated novel neurotrophin-1/B-cell-stimulating factor-3 [Nnt-1/BSF-3]).
- Gp130, membrane-spanning 130-kDa glycoprotein; GPI, glycosylphosphatidylinositol-anchored CNTF receptor α (Cnfrα); LIFR, LIF receptor; RT-PCR, reverse transcriptase polymerase chain reaction.

### References

Nobbio et al. J Neuropathol Exp Neurol • Volume 68, Number 5, May 2009
It is, therefore, essential to use a systematic sampling technique. A black mask with 5 holes (2 × 2.5 mm each) was superimposed on the slide, with the center of the mask overlying the center of the culture. From each of the 5 holes, at least 25 images were alternatively collected using the 20× objective and stored to save approximately 125 fields from each DRG culture. Over each digitized image, a grid mask made of 20 circles having an area of 1962.5 mm² each was superimposed by the Image Pro-Plus Software (Immagini e Computer). Then, only the myelinated segments contained in each circle were counted, and the total number was divided by the total examined area to obtain the number of myelinated segments per millimeter squared. This technique proved to be effective in distinguishing CMT1A from wild-type cultures (see Results section).

### Statistical Analysis

Results are presented as mean ± SEM of 3 separate experiments performed on different rat litters. Each condition was at least triplicated in each experiment. Multiple group comparisons of the differences in quantitative measurements were made by analysis of variance followed by a Dunnett posttest. Correlation analyses to plot g-ratios as a function of axonal diameter were performed using the Spearman rank correlation test. Finally, in the rrCNTF treatment experiments of DRG cultures, a two-tailed t-test for paired samples was used; p < 0.05 was considered to be significant.

### RESULTS

**CMT1A SCs Are Unable to Produce and Release CNTF (In Vitro and In Vivo)**

We first confirmed at the protein level that CNTF expression was markedly decreased in sciatic nerves of CMT1A rats (Fig. 1A), as well as in Pmp22²⁵ mice (Fig. 1B). To determine whether altered expression of CNTF is limited to experimental models of CMT1A as previously suggested (11–13), we evaluated CNTF levels in Pmp22⁻/⁻ and P₀⁻/⁻ mice, models of hereditary neuropathy with liability to pressure palsies (HNPP) and CMT1B, respectively. CNTF levels were unchanged or even increased in sciatic nerves of Pmp22⁻/⁻ and P₀⁻/⁻ mice compared with their internal controls (Pmp22 wt and P₀ +/+ mice, respectively) (mean ± SEM: 3,431 ± 31.10 vs 3,488 ± 37.85 pg; n = 4; p = ns and 3,511 ± 44.46 vs 3,055 ± 68.53 pg; n = 3; p < 0.05, respectively). Data were analyzed by a two-tailed t-test for unpaired samples.
Because altered expression of Cntf in the sciatic nerves of CMT1A rats is not due to rarefaction of SCs (11), we studied the ability of SCs to produce and release this trophic factor in culture. In absolute values, CNTF expression was significantly reduced in CMT1A SCs compared with controls. The difference was even more marked in SCs from homozygous than in SCs from heterozygous animals. In the supernatants of SC cultures, both homozygous and heterozygous SCs release significantly lower amounts of CNTF compared with controls (Table 2).

To test the ability of CMT1A SC to produce and release CNTF in vivo, we performed crush injuries on the sciatic nerves of heterozygous CMT1A rats and their wild-type littermates. Immunohistochemistry was used to localize CNTF in myelinating SCs. As expected, relative levels of Cntf mRNA and protein were significantly reduced in unlesioned CMT1A nerves compared with normal controls. One week after crush, there was a dramatic decrease in Cntf mRNA expression both in normal and affected nerves (Fig. 2A). After a recovery period of 3 months, both wild-type and transgenic animals reexpressed Cntf mRNA, but the remaining levels in CMT1A rats were significantly lower than in controls (Fig. 2B). As previously demonstrated (11), levels of CNTF protein were also significantly lower in unlesioned CMT1A nerves than in the wild-type littermates (Figs. 2C, D). One week postnerve crush, both control and transgenic rats showed evidence regeneration (Figs. 3B, D). In line with this observation, the frequency distribution of myelin thickness, axonal diameter, and fiber diameter became similar in wild-type and CMT1A rats after crush (Figs. 3B1, D1).

**TABLE 3. Morphometric Analysis of Charcot-Marie-Tooth Disease Type 1A and Wild-Type Rats**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fiber Density, fibers/mm²</th>
<th>Myelin Thickness, μm</th>
<th>Axon Diameter, μm</th>
<th>Fiber Diameter, μm</th>
<th>g-ratio</th>
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<tr>
<td>Wt A</td>
<td>0.0046 ± 0.00024</td>
<td>1.537 ± 0.39</td>
<td>5.369 ± 1.2</td>
<td>8.442 ± 1.99</td>
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<tr>
<td>Wt B</td>
<td>0.008 ± 0.00183</td>
<td>0.8830 ± 0.15</td>
<td>3.475 ± 0.89</td>
<td>5.241 ± 1.13</td>
<td>0.6408 ± 0.007</td>
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<td>CMT1A C</td>
<td>0.0058 ± 0.00056</td>
<td>1.315 ± 0.19</td>
<td>4.247 ± 0.86</td>
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<tr>
<td>CMT1A D</td>
<td>0.009 ± 0.00113</td>
<td>0.328 ± 0.24</td>
<td>2.891 ± 0.78</td>
<td>4.865 ± 0.87</td>
<td>0.5715 ± 0.015</td>
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Data were analyzed by one-way analysis of variance (ANOVA), followed by a Dunnett posttest to compare both wild-type (Wt) crushed nerves and Charcot-Marie-Tooth disease type 1A (CMT1A). Crushed and unlesioned nerves to the unlesioned wild-type ones.

**CNTF Expression Decreases With Age in Parallel With Molecular Indicators of Axonal Impairment in Heterozygous CMT1A Rats**

Postnatal days 3, 30, and 320 were arbitrarily chosen as representative of developmental, adult, and aging stages of rats. Relative levels of CNTF protein were significantly lower in sciatic nerves of CMT1A compared with wild-type rats at all the stages analyzed; the most significant difference became evident during the first month of age (Figs. 4A, B). Expression of nonphosphorylated NFs and APP were evaluated by Western blotting at the same time points as molecular markers of axonal impairment (24). Levels of nonphosphorylated NF were similar in CMT1A and wild-type rats in the early stages of development, but in parallel with the reduction in CNTF expression, there was a significant increase of nonphosphorylated NF both at P30 and P320 in the transgenic rats compared with normal controls (Fig. 4C). Amyloid precursor protein A4 behaved slightly differently in that it was significantly increased in affected animals only at P320 when the differences in terms of CNTF and nonphosphorylated NF were highest between the 2 groups (Fig. 4D).

**STAT3-GFAP Molecular Pathway in CMT1A**

To evaluate the activation of the molecular pathway downstream of the CNTF receptor, we measured mRNA expression of the Cntf receptor subunits both in sciatic nerves

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and in primary SC cultures. Protein levels and localization of STAT3 and GFAP were also determined. There was a significant upregulation of the complete Cntf receptor complex both in heterozygous and homozygous CMT1A nerves (Table 4). The same overexpression was also found in heterozygous and homozygous SC cultures (Table 4). When we analyzed the levels of mRNA for the Cntf-related family of cytokines, we did not observe any modulation of expression either in sciatic nerves or in SC cultures (Table 4).

In conjunction with upregulation of the Cntf receptor complex, we found significantly greater levels of Tyr705-pSTAT3 in homozygous and heterozygous CMT1A rats compared with wild-type controls (Fig. 5A). Moreover, a significant upregulation and hyperactivation of STAT3 was also observed in primary SC cultures (Fig. 5B). To determine whether these observations relate to myelinating or non-myelinating SC, we performed immunohistochemistry and immunofluorescence on CMT1A sciatic nerves and primary
FIGURE 3. Semithin sections of sciatic nerve (Toluidine blue; scale bars = 10 μm) from unlesioned wild-type (A) and Charcot-Marie-Tooth disease type 1A (CMT1A) (C) rats compared with postcrush (3 months) wild-type (B) and CMT1A (D) animals. Both wild-type and CMT1A nerves showed regeneration after crush (B, D). (A1-D1) Frequency histograms of myelinated fiber (MF) diameter (black bars), axonal diameter (white bars), and myelin thickness (dotted bars). Morphometric analysis was performed in wild-type (n = 3) and CMT1A (n = 3) rats on at least 300 MFs per animal from both unlesioned and crush-lesioned nerves. Unlesioned nerves from wild-type animals (A1) showed a bimodal distribution of MF diameter, whereas CMT1A rats displayed only one peak of MF diameter at 7 μm and a lack of larger fibers (>8 μm) (C1). At 3 months postcrush, the frequency distribution of myelin thickness, MF, and axonal diameter in CMT1A rats (D1) became similar to those of the wild-type rats (B1). g-ratio measurements in unlesioned CMT1A rat sciatic nerves showed hypermyelination of smaller and hypomyelination of larger axons (C2) compared with the wild-type rats (A2). Data were analyzed by one-way analysis of variance, followed by a Dunnett posttest to compare both wild-type crushed nerves and CMT1A unlesioned and crushed nerves to the unlesioned wild-type nerves. Correlation analyses to plot g-ratios as a function of axonal diameter were performed using the Spearman rank correlation test.
SCs (Fig. 6). We found that STAT3 (Fig. 6, Panel A) is expressed mainly in myelinating SC of both wild-type and CMT1A sciatic nerves; nuclear localization of this transcription factor was only seen in the transgenic animals. Consistent with this observation, pSTAT3 immunofluorescence detection on primary SC (Fig. 6, Panel B) showed marked hyperactivation in CMT1A SC compared with the controls. Finally, GFAP expression was evaluated by Western blot in CMT1A animals and immunohistochemistry in sural nerves of CMT1A patients (Fig. 6, Panel C). Corresponding to the upregulation and activation of STAT3, there were increased levels of GFAP in the CMT1A rats (Fig. 6, Panel C). Interestingly, a nerve biopsy from a CMT1A patient also showed greater immunoreactivity for GFAP in SCs (particularly of the nonmyelinating or denervated type and of those forming onion bulbs) than normal controls (Fig. 6, Panel C); this confirms our previous results (25).

**Administration of CNTF to Myelinating DRG Cultures Reduces Dephosphorylation of NFs in 30-Day-Old CMT1A Cultures**

We first evaluated the rate of myelination in wild-type and CMT1A DRG cultures (Fig. 7). As expected, the number of myelinated segments per millimeter squared was significantly greater in all wild-type cultures compared with the affected ones (p < 0.05; Fig. 7). We also exposed heterozygous CMT1A DRG cultures to rCNTF and to a vehicle control. Administration of rCNTF to CMT1A cultures did not improve the rate of myelination in any of the treated
cultures. On the contrary, levels of nonphosphorylated NFs were slightly but significantly reduced by treatment with rrCNTF in transgenic cultures. Treatment of wild-type cultures did not affect expression of nonphosphorylated NFs (Fig. 8).

### DISCUSSION

Ciliary neurotrophic factor was initially identified as a survival factor for chick ciliary neurons (26); it belongs to the IL-6 family of structurally related hematopoietic and neuropoietic cytokines (27, 28). Cellular responses to CNTF and

![Figure 5](http://jnen.oxfordjournals.org/)

**Figure 5.** Signaling cascade downstream of ciliary neurotrophic factor (CNTF) receptor. (A, B) Representative immunoblots (IB). Western blot analyses of STAT3 expression show that in both homozygous (+/+) and heterozygous (+/-) Charcot-Marie-Tooth disease type 1A (CMT1A) sciatic nerves (A) and Schwann cells (SCs) (B), STAT3 is overexpressed compared with the wild-type (wt) controls (sciatric nerves: mean ± SEM: 21.51 ± 3.984 and 7.76 ± 1.963, respectively, vs 4.5 ± 2.367; n = 3; analysis of variance [ANOVA], p < 0.01 and p = ns, respectively) (SCs: mean ± SEM: 42.50 ± 17.50 and 38.50 ± 3.50, respectively, vs 17.50 ± 3.50, respectively; n = 3; ANOVA, p = ns and p < 0.05, respectively) despite low levels of CNTF, but in accordance with Cntf receptor complex upregulation. Moreover, there is significantly increased activation of STAT3 (pSTAT3) in +/- and +/- CMT1A rats (A) and SCs (B) compared with wt controls (siatric nerves: mean ± SEM: 23.73 ± 2.309 and 7.96 ± 1.097, respectively, vs 1.1 ± 0.3464; n = 3; ANOVA, p < 0.001 and p < 0.01, respectively) (SCs: mean ± SEM: 17.50 ± 2.50 and 22.50 ± 2.50, respectively, vs 0.75 ± 0.25; n = 3; ANOVA, p < 0.05 for both comparisons). As a result, there is increased STAT3 tyrosine phosphorylation (pSTAT3/STAT3) in both +/- and +/- CMT1A rats (A1) and SCs (B1) compared with the wt controls (pSTAT3/STAT3 in sciatic nerves: mean ± SEM: 1.3 ± 0.1732 and 1.1 ± 0.6 vs 0.3464 ± 0.1135; n = 3; ANOVA, p < 0.05 and p = ns, respectively) (pSTAT3/STAT3 in SCs: mean ± SEM: 0.4650 ± 0.1350 and 0.5800 ± 0.01 vs 0.12 ± 0.02; n = 3; ANOVA, p = ns and p < 0.05, respectively).

### Table 4

<table>
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<th>Wild-Type Sciatic Nerve</th>
<th>+/- Sciatic Nerve</th>
<th>+/- Sciatic Nerve</th>
<th>Wild-Type Schwann Cells</th>
<th>+/- Schwann Cells</th>
<th>+/- Schwann Cells</th>
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<td>Gpi130</td>
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<td>2.4 ± 0.2*</td>
<td>3.2 ± 0.15*</td>
<td>0.343 ± 0.098</td>
<td>1.2 ± 0.15*</td>
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<td>Lifr</td>
<td>1.64 ± 0.338</td>
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<td>0.408 ± 0.124</td>
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<td>Cntf</td>
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<td>0.233 ± 0.140</td>
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<td>1.23 ± 0.273</td>
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<td>Clc</td>
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<td>0.09 ± 0.0024</td>
<td>0.299 ± 0.250</td>
<td>0.88 ± 0.235</td>
<td>0.88 ± 0.135</td>
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IL-6 type cytokines are elicited by different multiunit receptor complexes that always include the Gp130 subunit (29). Binding of CNTF to the GPI-anchored CNTFRα induces heterodimerization of the signal transducing β-receptors Gp130 and leukemia inhibitory factor receptor, which triggers intracellular signaling cascades mainly via the activation of Janus kinases and transcription factors of the STAT family (30). Ciliary neurotrophic factor exerts trophic support on motor and sensory neurons and on glial cells during development (31). It also acts as a lesion factor in the peripheral nervous system after injury (32). We and other authors previously demonstrated reduced expression of CNTF in experimental models of CMT1A, in the Trembler mouse and in human CMT1A nerves (11–13). In principle, downregulation of the Cntf gene might account for some features of CMT1A, including hypomyelination, secondary axonal damage, and muscle atrophy. Several questions, however, remain to be answered: 1) Is downregulation of CNTF specific to CMT1A among hereditary neuropathies? 2) Are CMT1A SCs congenitally unable to produce and release CNTF? 3) Does CNTF downregulation correspond to the development of axonal damage in CMT1A? 4) Can administration of CNTF prevent demyelination or axonal impairment in experimental CMT1A?

Our present results indicate that CNTF expression is impaired in nerves of CMT1A rats and mice but not in other models of hereditary neuropathies. Production and release of CNTF are also affected in cultures of CMT1A SCs. The crushed sciatic nerve experiments indicate that CMT1A SCs produce lower levels of CNTF compared with wild-type cells in vivo. Moreover, CNTF expression in sciatic nerves of CMT1A rats decreases significantly with increasing age. As expected, molecular markers of axonal damage (i.e. dephosphorylation of NFs and expression of APP) increase with age, thus paralleling the progression of CNTF downregulation. Finally, the treatment of CMT1A DRG cultures with rrCNTF reduces dephosphorylation of NFs without affecting myelination.

**FIGURE 6.** Signal transducer and activator of transcription 3 (STAT3) overexpression and hyperactivation in Charcot-Marie-Tooth disease type 1A (CMT1A) nerves and Schwann cells (SCs). **Panel A:** Immunohistochemistry showing STAT3 expression in myelinating SCs of wild-type (Wt) animals (A). In CMT1A sciatic nerves (B), there also is some expression in nonmyelinating SCs (arrowheads), but the protein localization is different in the 2 conditions; it is mainly cytoplasmic in Wt cells (A, arrows) and more nuclear in the transgenic nerves (B, arrows). **Panel B:** Immunofluorescence detection of pSTAT3 in primary SCs shows increased expression of this transcription factor in nuclei that are counterstained with 4',6-diamidino-2-phenylindole (DAPI) (C, F) of CMT1A (+/−) SCs (G) compared with control (Wt) SCs (D). **Panel C:** Increased levels and activation of STAT3 are coupled with overexpression of glial fibrillary acidic protein (GFAP) in CMT1A rats by Western blot. Immunohistochemistry on a human sural nerve biopsy of a CMT1A patient (L) compared with a normal control (I) shows prominent immunostaining in the CMT1A patient nerve. Schwann cells of the nonmyelinating or denervated type (arrows) and SCs forming onion bulbs (open arrow) show GFAP immunostaining. 2,3-Diaminobenzidine; scale bars = 10 μm.)
FIGURE 7. Myelinating dorsal root ganglia cultures from 15-day-old Charcot-Marie-Tooth disease type 1A (CMT1A) and wild-type embryos after 30 days in vitro. (A) Most fibers in CMT1A cultures show axonal segments devoid of myelin along with normally myelinated internodes (arrows). (B) Normal cultures containing numerous myelinated fibers with normal nodes of Ranvier. (C) Morphometric evaluation of myelination rate shows that the number of myelinated segments per millimeter squared is significantly greater in all wild-type (wt) cultures compared with affected (+/−) cultures. Control cultures (n = 5) ranged from 0.00040 to 0.00094 myelinated segments/mm² (mean ± SEM: 0.000728 ± 0.00009); CMT1A cultures (n = 9) showed a maximum of 0.00036 myelinated segments/mm², with most cultures having less than 0.00020 myelinated segments/mm² (mean ± SEM: 0.0002089 ± 0.00003). (Osmium tetroxide and Sudan black; scale bars = 10 μm). Data were analyzed by a two-tailed t-test for unpaired samples.

Schwann cells are a well-known source of trophic factors for axons (33). In particular, myelinating SCs express high amounts of CNTF, and expression of CNTF in SCs is regulated by axonal contact (34). Specific and finely tuned regulation of trophic factors production by SCs has been observed after sciatic nerve damage (35). Because it is possible that SCs need to acquire a stable myelinated phenotype to express CNTF at the maximum levels (34), we assessed CNTF expression in other models of hereditary dysmyelinating neuropathies to determine whether the downregulation of CNTF could be merely due to the myelinopathy. The observation of normal or even increased levels of CNTF in experimental HNPP and CMT1B (Pmp22+/− and Po−/− mice, respectively) strongly suggests that the CMT1A SCs have a specific inability to express this trophic factor. Indeed, as noted above, CMT1A SCs significantly produced and released less CNTF into the supernatant than control SCs in vitro. The possibility that nonglial cells contribute to the downregulation of CNTF in CMT1A nerves was excluded by immunohistochemistry because only myelinating SCs showed reduced CNTF expression in sciatic nerves of affected rats. Moreover, it is known that CNTF but not brain-derived neurotrophic factor or other neurtrophins are downregulated in CMT1A nerves and in the Trembler mouse (11, 13). Nerve crush experiments also demonstrated that CMT1A SCs produce lower amounts of CNTF than wild-type SCs in vivo. As expected, at 1 week postcrush, there was downregulation of CNTF mRNA and protein in both CMT1A and wild-type rat nerves (32). At 3 months postcrush, CMT1A nerves again expressed significantly lower levels of CNTF mRNA and protein compared with wild-type littermates. At this time point, however, there was a dichotomy between mRNA and protein levels in both wild-type and transgenic rats. To explain this result, we speculate that the protein is not produced locally by the SCs but is transported from the periphery along the axons that have the potential for retrograde transport of CNTF (36). An increase of CNTF protein not originating from resident SCs may explain why we found morphological and morphometric signs of regeneration also in CMT1A nerves at the 3-month time point. In fact, the density of myelinated fibers in CMT1A nerves was similar to that of controls, whereas myelin thickness and axon diameter remained lower in affected than in wild-type nerves. Interestingly, the g-ratio shows a further and significant decrease in transgenic nerves compared with the control ones, indicating that the axon counterpart was attenuated compared with the myelin component.

These results and the observation that CNTF expression significantly decreases with age in CMT1A rats suggest that the downregulation of CNTF may be one of the molecular events involved in the progressive axonal atrophy typical of human and experimental CMT1A (3, 4). Indeed, in our rat model, the reduction of CNTF strictly parallels the increase of nonphosphorylated NFs and APP, the molecular markers of axonal injury (24, 37), and it is known that CNTF is necessary for long-term maintenance of nerve fibers and axons (15). Finally, because CNTF also has a myotrophic function (38), its downregulation might explain the muscle atrophy typically found in this animal model and in human CMT1A (2, 9, 18).

To understand whether a downregulation of CNTF in CMT1A rats also affects the classical pathway through which this trophic factor exerts its protective effects, we analyzed the STA3/GFAP molecular cascade. In CMT1A sciatic nerves and SCs, there was a significant upregulation of the Cntf receptor itself and of the STA3-GFAP pathway. Whether nonglial cells contribute to this overexpression is difficult to establish based on the present data, but immunohistochemistry showed an overexpression and a translocation to the nucleus of STAT3 in myelinating SCs of CMT1A rats. This pattern of expression and activation was also observed in cultured SCs. Interestingly, human CMT1A nerves show an upregulation of GFAP, which involves SCs of the myelinating and denervated type (25). It is possible that the abnormal activation of STAT3 in CMT1A rats represents a consequence of the impaired differentiation program of CMT1A SCs (22, 39). This hypothesis is also supported by

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the upregulation of cyclin D1 previously found in CMT1A nerves (11, 40) because the constitutive activation of STAT3 plays a causative role in overexpression of this cell cycle protein (41). Induction of STAT3 signaling plays a key role in the development of reactive gliosis in the central nervous system after different types of damage (42), and we speculate that upregulation of the STAT/GFAP molecular cascade may contribute to the hypertrophy of SCs and onion bulb formation that are the neuropathological hallmarks of CMT1A.

In contrast to previous reports in different human peripheral neuropathies (43–46), downregulation of CNTF does not strictly correspond to a reduction of myelinated fibers in experimental CMT1A (present data and Reference [11]); homozygous nerves do, however, express the lowest levels of the trophic factor (11). This observation may also suggest that PMP22 overexpression reduces expression of the Cntf gene by influencing its transcription through still unidentified molecular mechanisms. To date, the literature does not address the question whether PMP22 has direct or indirect effects on the transcription of any gene; further studies are needed to clarify this point.

The above observations and the previously reported promyelinating effect of CNTF (17) prompted us to test the effects of CNTF in our in vitro model of CMT1A (23). Because one of the end points was to determine whether CNTF treatment could prevent demyelination, we performed morphometry at the light microscopic level and found a clear cutoff between affected and normal cultures (Fig. 7). Administration of rrCNTF to CMT1A cultures did not improve the rate of myelination in any of the treated cultures (mean number of myelinated segments/mm² ± SEM: Vehicle: 0.000193 ± 0.00003; n = 10 in vehicle treated cultures vs. 0.000207 ± 0.00003; n = 10 in rrCNTF-treated cultures; p = ns). Representative cultures are illustrated on the left. (B) Graph of Western blot analysis of SMI32 shows that chronic treatment with rrCNTF reduced this axon injury marker compared with vehicle-treated cultures. On average, expression of SMI32 was significantly reduced by treatment with rrCNTF in transgenic cultures from 3 different offspring (vehicle-treated: 1.154 ± 18.35 vs treated: 0.93 ± 0.65; n = 20; p < 0.05). Treatment of wild-type cultures with rrCNTF did not affect expression of SMI32 (0.59 ± 0.3 vs 0.56 ± 0.4; n = 7; p = ns). (Osmium tetroxide and Sudan black; scale bars = 10 μm). Data were analyzed by a two-tailed t-test for paired samples.
carrying \textsc{cntf} null alleles do not show a higher risk of developing hereditary neuropathies (e.g. CMT1A or HNPP, or amyotrophic lateral sclerosis [47]) does not exclude \textsc{cntf} as a gene that may modify the disease phenotype. In fact, mice lacking \textsc{cntf} develop severe axonal and muscle atrophy over time (15). Finally, the administration of \textsc{cntf} in our culture system reduces molecular features of axonal damage, indicating that further studies in the animal model are warranted to understand whether \textsc{cntf} or its derivatives may be considered as future therapies for CMT1A. Eventually, this may be in association with other agents, such as ascorbic acid or onapristone, which are able to reduce the expression of PMP22. Indeed, the neurophin 3 has shown promising results in the treatment of experimental and human CMT1A (48, 49).


does not exclude CMT1A (48, 49).

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REFERENCES


43. Ito Y, Yamamoto M, Mitsuma N, et al. Expression of mRNAs for ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and their receptors (CNTFR alpha, LIFR beta, IL-6R alpha, and gp130) in human peripheral neuropathies. Neurochem Res 2001;26:51–58